Novel haem co-ordination variants of flavocytochrome P450 BM3

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Bacillus megaterium flavocytochrome P450 BM3 is a catalytically self-sufficient fatty acid hydroxylase formed by fusion of soluble NADPH–cytochrome P450 reductase and P450 domains. Selected mutations at residue 264 in the haem (P450) domain of the enzyme lead to novel amino acid sixth (distal) co-ordination ligands to the haem iron. The catalytic, spectroscopic and thermodynamic properties of the A264M, A264Q and A264C variants were determined in both the intact flavocytochromes and haem domains of P450 BM3. Crystal structures of the mutant haem domains demonstrate axial ligation of P450 haem iron by methionine and glutamine ligands trans to the cysteine thiolate, creating novel haem iron ligand sets in the A264M/Q variants. In contrast, the crystal structure of the A264C variant reveals no direct interaction between the introduced cysteine side chain and the haem, although EPR data indicate Cys264 interactions with haem iron in solution. The A264M haem potential is elevated by comparison with wild-type haem domain, and substrate binding to the A264Q haem domain results in a ~360 mV increase in potential. All mutant haem domains occupy the conformation adopted by the substrate-bound form of wild-type BM3, despite the absence of added substrate. The A264M mutant (which has higher dodecanoate affinity than wild-type BM3) co-purifies with a structurally resolved lipid. These data demonstrate that a single mutation at Ala264 is enough to perturb the conformational equilibrium between substrate-free and substrate-bound P450 BM3, and provide firm structural and spectroscopic data for novel haem iron ligand sets unprecedented in Nature.

Key words: cytochrome P450, electron paramagnetic resonance (EPR), haem co-ordination, magnetic circular dichroism (MCD), P450 BM3.

INTRODUCTION

The cytochromes P450 (P450s) are a superfamily of mono-oxygenase enzymes responsible for a vast array of physiologically and biotechnologically important reactions in virtually all life forms [1,2]. Their catalytic repertoire includes such reactions as hydroxylation, dehalogeneration, dehydorination, epoxidation and isomerization [2,3]. Oxidative catalysis by P450s requires reduction of the resting (ferric) haem iron by two electrons: the first generating ferrous haem iron that binds molecular oxygen and the second reducing the ferrous oxy (or ferric superoxy) form to a ferric peroxy species. Successive protonations of this short-lived species generates the even more transient ferric hydroperoxy form (also known as compound 0) and (following its dehydorination) the ferryl oxy (or compound 1) intermediate [4]. Compound 1 is considered to be the ultimate oxidant of the substrate in the vast majority of P450-dependent reactions, although it has proved elusive to isolation and characterization [5,6]. Cryogenic spectroscopic studies have, however, provided conclusive evidence for the formation of the compound 0 intermediate [7]. The electrons required for P450 catalysis are usually donated by NAD(P)H coenzymes and delivered to the P450 by one or more redox partner enzymes [6]. A typical mammalian P450 system consists of the FAD- and FMN-binding NADPH-dependent CPR (cytochrome P450 reductase) and the P450, both of which are membrane-bound proteins [8]. A typical prokaryotic P450 system (e.g. the Pseudomonas putida camphor hydroxylase P450cam) has soluble FAD-binding and NAD(P)H-dependent ferredoxin reductase, ferredoxin and P450 components [9]. However, it is now recognized that there is considerable biological diversity in the types of P450 redox apparatus. For example, P450 systems exist that have CPR fused to the P450, use pyruvate-dependent reductase partner proteins, or have dispensed altogether with redox partners in favour of direct interactions with peroxide or NAD(P)H [6,10].

One of the best characterized P450s, and an example of the class in which P450 and CPR-type enzymes are fused in a single polypeptide, is flavocytochrome P450 BM3 from the soil bacterium Bacillus megaterium [11]. BM3 uses NADPH-derived electrons to catalyse hydroxylation of fatty acids, and has the highest mono-oxygenation rate yet reported for a P450 enzyme [12,13]. Protein engineering has been used extensively to explore structure–function relationships in BM3, to facilitate domain dissection and kinetic/thermodynamic characterization and to modify its substrate recognition to enable oxidative catalysis of different substrates for biotechnological exploitation (e.g. alkanes and styrene [14–17]).

Factors for consideration in the exploitation of P450s for large-scale production of oxyfunctionalized organic molecules is their inactivation by loss of haem iron ligation and/or displacement of the haem macrocycle altogether from the protein matrix [11,18]. In this respect, the demonstration that certain eukaryotic fatty acid hydroxylase P450s (CYP4 family) have their haem covalently attached (via a haem methyl group) to an acidic active-site residue (usually glutamate) was an exciting discovery, and mechanistic studies have demonstrated that the esterification reaction occurred in a P450-turnover-dependent manner [19,20]. The possibility that increased haem stability could be imparted on other P450s...
by engineering a glutamate residue in the appropriate active-site location was explored for the biotechnologically important P450cam and BM3 enzymes. A small amount of covalent linkage of haem to Glu264 through the haem 5-methyl group was induced through enzymatic turnover of a G248E mutant of P450cam, but this was not achieved for the comparable A264E mutant of BM3 [21,22]. However, in the case of BM3, the A264E mutant instead exhibited partial co-ordination of the Glu264 side chain as the distal ligand to the haem iron in the substrate-free form of the enzyme and essentially complete Glu264 co-ordination in the substrate (fatty acid)-bound form, with simultaneous retention of the cysteinate axial ligand (Cys400) on the proximal side of the haem [22,23]. In the light of these data, we went on to create A264H/K mutants in the BM3 haem (P450) domain, where the His264 and Lys264 side chains were shown to co-ordinate to the haem iron fully in both substrate-free and fatty-acid-bound forms of the protein [24]. It was clearly established that point mutants at Ala264 offered the opportunity to create different types of haem iron ligand sets, including variations not observed to date in Nature. In the present work, the properties of A264M, A264C and A264Q mutants of BM3, exploiting spectroscopic methods and demonstrating further novel haem ligand sets. In addition, we have characterized the status of the proximal haem ligand (thiol compared with thiolate), the properties of the reduced A264M/C/Q/H/E enzymes have been investigated for the first time, and crystal structures for the A264M/C/Q variants are presented for these novel haem co-ordination mutants. Our data reveal further new forms of proteinaceous haem co-ordination and spectroscopic signatures for these species.

**EXPERIMENTAL**

**Materials**

Restriction enzymes were from NEB. Ampicillin, NADPH, yeast extract and tryptone were from Melford Laboratories. DEAE-Sephrose Fast Flow and Q-Sepharose were from GE Healthcare. Ceramic hydroxypatite was from Bio-Rad Laboratories. PEG [poly(ethylene glycol)] 3350 and PEG 200 were from Fluka. CO and NO were from BOC Gases. All other reagents were purchased from Sigma–Aldrich and were of the highest grade available.

**Mutagenesis, gene expression and protein purification**

Generation of the A264K and A264H mutants in both haem domain and intact flavocytochrome constructs has been described elsewhere [24]. The A264C, A264M and A264Q mutations were each generated in both haem domain (pBM20) and flavocytochrome (pBM25) constructs using the Stratagene QuikChange® mutagenesis kit. The primers used are described in Table 1. Entire genes were sequenced to verify the presence of the desired mutation and to confirm that no further unwanted mutations were present. The mutant and WT (wild-type) genes were expressed and proteins were purified as described previously for both the haem (P450) domain and intact flavocytochrome forms of the enzyme [13,14,25].

**Spectroscopic analysis**

Electronic absorption spectroscopy

UV–visible absorption spectra for the BM3 proteins were collected using a Cary 50 scanning spectrophotometer (Varian) and using a 1-cm-pathlength quartz cuvette. Haem concentrations were calculated for each P450 mutant and WT protein using the pyridine haemochromagen method [26]. Spectra were collected for WT and mutant enzymes in the oxidized, dithionite-reduced, reduced CO-bound, substrate-bound and inhibitor-bound states, as described previously [27]. Enzyme concentration for LS (low-spin) WT BM3 haem domain and flavocytochrome forms were determined spectrophotometrically using the previously established molar absorption coefficients of $ε_{max} = 95$ and 105 mM$^{-1}$ cm$^{-1}$ respectively [13]. Binding of fatty acids and azole inhibitors to WT P450 BM3 and Ala264 variants was measured by spectral titration as described previously [22]. Binding titration data collection and fitting methods are described in greater detail in the Supplementary Online Data at http://www.BiochemJ.org/bj/417/bj4170065add.htm.

Electronic paramagnetic resonance

EPR spectra were collected using an EPR spectrometer comprising an ER200D electromagnet and microwave bridge interfaced to a EMX control system (Bruker Spectrospin), and fitted with a liquid helium flow cryostat (ESR-9, Oxford Instruments) and a dual-mode X-band cavity (Bruker type ER4116DM). Spectra were recorded for WT (400 μM), A264C (450 μM), A264M (520 μM) and A264Q (435 μM) haem domains in assay buffer at 10.8 K. EPR data for the A264E/K/H mutants were reported previously [22–24].

MCD (magnetic CD)

MCD spectra were recorded using JASCO J/810 and J/730 dichrographs in the near-UV–visible and NIR (near-IR) regions respectively using an Oxford Instrument superconducting solenoid with a 25 mm ambient bore to generate a magnetic field of 6 T. A 0.1-cm-pathlength quartz cuvette was used to record NIR spectra with sample concentrations the same as those used for EPR spectral collection. UV spectra were recorded for WT (30 μM), A264M (130 μM), A264Q (85 μM) and A264C (90 μM) haem domains with 50 mM Hepes in $^3$H$_2$O (pH 7.0) as buffer (where pH$^*$ is the apparent pH measured in $^3$H$_2$O using a standard glass pH electrode).

RR (resonance Raman)

RR spectra were collected for substrate-free and arachidonate-bound (500 μM) WT, A264Q, A264M and A264C mutant haem domains (50 μM) at ambient temperature. A 15 mW 406.7 nm

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### Table 1 Creation of A264 mutants

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>A264MF</td>
<td>5′-CATCTTAATTAGTGGCATTAGAACAACAGTG3′-</td>
</tr>
<tr>
<td>A264MR</td>
<td>5′-CCACTGTTTCCATAGACCATATTGAGAGT3′-</td>
</tr>
<tr>
<td>A264OF</td>
<td>5′-CATCTTAATTAGTGGCATTAGAACAACAGGG3′-</td>
</tr>
<tr>
<td>A264OR</td>
<td>5′-CCACTGTTTCCATAGACCATATTGAGAGT3′-</td>
</tr>
<tr>
<td>A264CF</td>
<td>5′-CATCTTAATTAGTGGCATTAGAACAACAGTG3′-</td>
</tr>
<tr>
<td>A264CR</td>
<td>5′-CCACTGTTTCCATAGACCATATTGAGAGT3′-</td>
</tr>
</tbody>
</table>

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radiation source was used at the sample and delivered from a Coherent Innova 300 krypton ion laser, with spectra acquired with a Renishaw micro-Raman system 100 spectrometer. The samples were held in a capillary under a microscope with five 15 s exposures in each case.

**Kinetic studies and product analysis**

Steady-state kinetic studies of P450 BM3 substrate-dependent NADPH oxidation were carried out with WT and each mutant flavocytochrome using arachidonate and dodecanoate as substrates, as described previously [13]. Activities of the reductase domains of the flavocytochromes were analysed by cytochrome c reduction assays, as described previously [13]. Analysis of the turnover of dodecanoate was carried out in 100 mM potassium phosphate buffer (pH 7.0) at 25 °C using 400 nM WT and Ala264 mutant BM3 enzymes, 600 μM NADPH and 45 μM dodecanoate with continuous stirring. Reactions were carried out in duplicate with control samples without enzyme added. Following complete oxidation of NADPH, the reaction mixture was adjusted to pH 2 by addition of 1 M HCl. Thereafter, lipids were extracted into dichloromethane and water was removed by addition of solid magnesium sulfate. The filtered solution was then evaporated to dryness, and the lipids were dissolved in methanol, with sample analysis and quantification performed using a Micromass Quattro triple quadrupole mass spectrometer, as described in our previous studies [28].

**Potentiometric analysis**

Potentiometric titrations were carried out using the spectroelectrochemical method of Dutton and as described previously, with 6–10 μM enzyme in a 5 ml sample volume [29,30]. Redox potentiometry was carried out in a Belle technology glove box under a nitrogen atmosphere and with oxygen levels maintained at less than 2 p.p.m. In order to remove residual traces of oxygen, protein samples were passed through a Bio-Rad 10DG gel-filtration column equilibrated with redox buffer (100 mM potassium phosphate, pH 7.0, containing 10% glycerol). Potentiometry data collection and fitting methods are described in full in the Supplementary Online Data at http://www.BiochemJ.org/bj/417/bj4170065add.htm.

**Structural studies**

The A264C, A264M and A264Q haem domains were crystallized using the sitting-drop technique at 4 °C. Drops were prepared by addition of 2 μl of mother liquor to 2 μl of 12 mg/ml protein solution in 10 mM Tris/HCl at pH 7.5. A264Q crystals were obtained with a mother liquor of 100 mM cacodylic acid (pH 6.0), 160 mM MgCl₂ and 16% PEG 3350. A264M crystals were obtained with mother liquor of 100 mM cacodylic acid (pH 6.0), 140 mM MgCl₂ and 18% PEG 3350. A264C crystals were obtained under the same conditions, but with 100 mM MgCl₂. Crystals were flash-frozen in liquid nitrogen using 10% PEG 200 as cryoprotectant. Structures of the A264E/H/K mutants were reported previously [22–24].

Diffraction data for the A264C, A264M and A264Q mutants was collected on single flash-cooled crystals at ESRF ID14 beamlines (European Synchrotron Radiation Facility, Grenoble, France). Data were analysed and merged using MOSFLM and SCALA [31]. The A264E structure was used as a starting model, with positional and B-factor refinement using REFMAC5 [32] alternated with manual model-building cycles using COOT [33]. Final refinement and data collection statistics are given in Supplementary Table S1 at http://www.BiochemJ.org/bj/417/bj4170065add.htm.

**RESULTS AND DISCUSSION**

**Expression and purification of P450 BM3 haem co-ordination variants**

Previous studies of the P450 BM3 A264E variant indicated that this I-helix mutation did not significantly affect expression/purification of the mutant BM3 flavocytochrome or haem domains [22,23]. The A264C/H/K/M/Q haem domains and flavocytochromes were expressed in *Escherichia coli* as described previously for WT P450 BM3 and the A264E variant [14,22,23]. A264M/C/Q mutations did not result in notable decreases in expression of the respective haem domains or flavocytochromes. The intact (flavocytochrome) forms of the A264K/H mutants were similarly unaffected. Enzymes were purified to homogeneity using chromatographic methods established for the WT enzyme (DEAE-Sepharose, Q-Sepharose and hydroxyapatite resins). Protein yields were ~ 50 mg/l for the haem domains and ~ 30 mg/l for the intact enzymes. Concentrations of the mutant P450s were determined as described previously, or using molar absorption coefficients of 97, 113 and 98 mM⁻¹·cm⁻¹ for the A264Q/C/M proteins respectively [13,24].

**UV-visible absorption properties of P450 BM3 Ala264 mutants**

Oxidized spectra

In all mutants, the haem spectral properties in the haem domains were indistinguishable from those of the flavocytochrome forms (i.e. those with appended diflavin reductase domain) and thus optical data described here pertain to haem domains. The UV–visible absorption spectrum of a P450 is a sensitive indicator of haem iron oxidation and spin state, and is informative regarding changes in co-ordination of haem iron in the distal position (i.e. the loss of an axial water as the haem iron sixth ligand, or its replacement with another ligand). Previous studies of the A264E variant indicated partial occupancy of the sixth co-ordination position on the BM3 haem iron by the Gln390 side chain in the substrate-free enzyme (Soret maximum at 420.5 nm compared with ~418 nm for WT haem domain), and a near-complete conversion into a Cys-Fe-Glu ligand set on saturation with fatty acid substrates (Soret at 426 nm) [22]. The Soret maxima for the A264K/H mutants of the ferric haem domains are at 424/427 nm, consistent with complete distal co-ordination of the haem iron by the Lys/His394 side chains, as observed in the relevant haem domain crystal structures [24].

For the A264Q haem domain, spectral features of the oxidized ligand-free enzyme were similar to those for WT BM3, with the Soret band at 418 nm, and α/β bands at 569/534 nm (compared with 569/535 nm for WT BM3), suggesting a WT-like co-ordination state of the haem iron. However, spectroscopic studies indicated at least partial co-ordination of haem iron by the Gln394 side chain and that similarities with the WT oxidized spectrum might originate from the nature of the novel ligation state (i.e. via the amide oxygen atom, see below). The A264C haem domain has a spectrum near-identical with that of WT haem domain, with maxima at 418, 569 and 535 nm. A more pronounced shoulder on the low-wavelength side of the Soret band (at ~390 nm) suggested a greater HS (high-spin) haem iron content than in WT (Figure 1). The A264M haem domain exhibited spectral maxima at 416, 566 and 534 nm. A more prominent shoulder (than that of the WT) was again observed at ~390 nm, consistent with more HS ferric haem iron. This probably reflects dissociation of the

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The absence of a red-shifted Soret band in the A264C/M mutants (as seen in A264K/H/E variants) does not suggest novel haem iron co-ordination states in these mutants. However, structural and spectroscopic studies indicated that, at least for the A264M mutant, the Met\textsuperscript{264} side chain co-ordinates the BM3 haem iron (see below).

### Fatty acid substrate binding

Previous work showed fatty acid substrate-dependent formation of the Cys-Fe(III)-Glu ligand set in the A264E BM3 mutant [22]. Structural studies showed that, in substrate-free enzyme, the Glu\textsuperscript{264} side chain either (i) co-ordinates haem iron in the sixth position, or (ii) interacts with the phenyl group of Phe\textsuperscript{87} in the pocket above the haem plane. Fatty acid binding displaces the Glu\textsuperscript{264} side chain from the Phe\textsuperscript{87} phenyl and results in near-complete co-ordination of the haem iron [22]. In contrast, fatty acid binding (e.g. arachidonate, palmitoleate or dodecanoate) did not affect the (already hexa-co-ordinated) ligation state of the A264K/H mutants. For A264Q haem domain, fatty acid binding resulted either in negligible optical perturbation (dodecanoate) or in minor shifts indicating accumulation of HS haem iron (for palmitoleate and arachidonate). For longer-chain fatty acids, the $K_d$ values computed from optical titrations were similar to those for WT haem domain (e.g. 6.43 ± 0.51 μM for palmitoleate compared with 3.50 ± 0.17 μM for WT), despite much lower overall conversion into the HS form (Table 2). These data indicated retention of high substrate affinity in the A264Q mutant, but pointed to a less efficient sixth ligand displacement by lipid substrates, and hence the possibility that the Gln\textsuperscript{264} side chain co-ordinates the haem iron in a proportion of the molecules. For A264C haem domain, fatty-acid-induced perturbation of the spin-state equilibrium was also observed in some cases, but the extent of HS conversion was substantially less than observed for WT with arachidonate ($K_d = 6.4 \pm 0.3$ μM compared with 0.55 ± 0.05 μM for WT) and palmitoleate, and negligible optical change was observed on titration with the saturated fatty acids palmitate (C\textsubscript{16}), myristate (C\textsubscript{14}) or dodecanoate (C\textsubscript{12}). For the A264M mutant, a more substantial fatty-acid-induced HS conversion of haem iron was observed than for A264C, albeit slightly less than for WT with the same fatty acids. $K_d$ determinations indicated a higher fatty acid affinity for A264M than for WT BM3 haem domain. For example, $K_d$ values for A264M (WT) were 7.4 ± 0.4 μM.

### Table 2 Kinetic parameters for fatty acid oxidation by WT and Ala\textsuperscript{264} mutant flavocytochromes P450 BM3

Data were collected as described in the Experimental section. The $k_{cat}$ data indicated with asterisks (*) are second-order rate constants expressed in units of μM\textsuperscript{-1}·min\textsuperscript{-1} with respect to fatty acid concentration. ND indicates ‘not determinable’ for mutants in which fatty acid binding did not induce any significant perturbation of haem iron optical properties. WT data for arachidonate oxidation kinetics are from [12]. Data for fatty acid oxidation kinetics and binding for the A264E mutant are from [22].

<table>
<thead>
<tr>
<th>BM3 enzyme</th>
<th>Fatty acid substrate</th>
<th>$k_{cat}$ (μM)$^*$</th>
<th>$K_m$ (μM)</th>
<th>$K_d$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>Dodecanoate</td>
<td>2770 ± 120</td>
<td>87.4 ± 8.1</td>
<td>89 ± 15</td>
</tr>
<tr>
<td></td>
<td>Arachidonate</td>
<td>0.25 ± 0.07</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>A264H</td>
<td></td>
<td>0.36 ± 0.07</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>A264K</td>
<td></td>
<td>1.92 ± 0.17</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>A264Q</td>
<td></td>
<td>195 ± 5</td>
<td>5.2 ± 0.8</td>
<td>17.8 ± 0.9</td>
</tr>
<tr>
<td>A264M</td>
<td></td>
<td>0.08 ± 0.01</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>A264C</td>
<td></td>
<td>744 ± 21</td>
<td>114 ± 12</td>
<td>ND</td>
</tr>
<tr>
<td>A264E</td>
<td></td>
<td>2770 ± 120</td>
<td>87 ± 8</td>
<td>100 ± 15</td>
</tr>
</tbody>
</table>

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(11.3 ± 0.4 μM) for palmitate, 0.9 ± 0.3 μM (6.9 ± 0.4 μM) for myristate and 17.8 ± 0.9 μM (89.0 ± 15.0 μM) for dodecanoate (Table 2). Thus selected fatty acids were able to induce partial haem iron sixth ligand displacement for both the A264C/M mutants.

Inhibitor binding

To compare further the optical and ligand-binding features of WT and Ala264 mutant enzymes, we analysed the haem domain interactions with an inhibitory azole ligand that co-ordinates the haem iron in the sixth (axial) position. We chose 4-PIM (4-phenylimidazole), a small azole ligand with high affinity for P450 BM3 [22]. 4-PIM binding produced a Soret shift to 424 nm in WT haem domain, consistent with co-ordination of an imidazole nitrogen to the haem iron, and with a $K_d = 0.84 ± 0.47$ μM. The binding of 4-PIM is substantially stronger than for imidazole itself ($535 ± 5$ μM), consistent with our recent studies of binding of these ligands to the Mycobacterium tuberculosis CYP51B1 sterol demethylase P450 (542 ± 27 μM for 4-PIM compared with $11.7 ± 0.9$ μM for imidazole) [34]. 4-PIM did not displace the endogenous amino acid sixth ligand in either A264H or A264K mutants. However, type II optical changes indicative of 4-PIM binding were observed for the other Ala264 mutants, consistent with the replacement of any aqua (or amino acid) ligand on the haem iron by the azole. For A264E haem domain, 4-PIM titration results in a Soret band shift to 426 nm (isosbestic point at 422 nm), with a $K_d = 2.51 ± 0.49$ μM. Our previous EPR studies indicated that the complex formed is homogeneous with 4-PIM co-ordinated completely to the haem iron [22]. For A264Q, the Soret shift is to 421 nm (isosbestic point at 424 nm), with a $K_d = 390 ± 27$ μM; for A264C the shift is to 422.5 nm (isosbestic point at 425.5 nm, $K_d = 52.0 ± 2.8$ μM); and for A264M the shift is to 420.5 nm (isosbestic point at 424 nm, $K_d = 440 ± 44$ μM). 4-PIM is not highly soluble in aqueous solution, and turbidity develops at > 2 mM. The 4-PIM $K_d$ values for A264M/C/Q, although within this concentration range, report on only a partial conversion into the 4-PIM–haem iron complex, as shown in Figure 2(A) for the A264C mutant. At higher 4-PIM concentrations (up to ~ 10 mM added), a distinct second phase of absorption change was observed for the A264C/Q mutants, indicating a secondary binding event of lower affinity. No further significant absorption shift was observed for the A264M mutant in this range.

Gaseous ligand binding

WT haem domain shows near-complete conversion into a ferrous–CO [Fe(II)CO] complex with spectral maximum at ~ 448 nm following bubbling with CO gas in the presence of sodium dithionite reductant. This confirms retention of a thiolate proximal ligand (from Cys60) in the Fe(II)CO complex, and negligible formation of a thiol-co-ordinated P420 species (Soret at 420 nm for BM3) [18,35]. Previous studies of A264K/H haem domains demonstrated negligible formation of a Fe(II)CO complex [24]. As discussed below, reduction of these domains to the ferrous form is feasible under anaerobic conditions, but the ferrous haemms retain their lysine/histidine distal ligands and there is no evidence for CO binding. In contrast, A264E haem domain underwent near-complete conversion into a Fe(II)CO complex with Soret maximum at 449 nm (P450 form), and only a small feature at 423 nm from a minor proportion (~ 10%) of P420 [22]. The Fe(II)CO complex of A264Q haem domain (Figure 2B) has its Soret maximum at 448 nm (shifted from 418 nm in the ferric, water ligated state), indicating near-complete formation of the P450 form. For A264M, the Fe(II)CO complex is again essentially completely P450 (Soret at 449 nm). For A264C, complete conversion into the Fe(II)CO species proved difficult, but the species formed was also almost completely P450 (Soret at 448 nm). None of the mutants exhibited any significant conversion of P450 to P420 (or vice versa) for up to 1 h, indicating stability of the proximal thiolate ligand [34–36].

Ferric P450s are known to complex with NO to form Fe(III)NO [formally Fe(II)NO+] adducts with distinctive spectral features [37]. For WT BM3 haem domain, the complex has its Soret band shifted to 434 nm, with complete conversion into the nitrosyl complex. The same is true for the A264E haem domain (Soret at 435 nm) [22]. As for the CO-binding data, NO complexes were not formed by A264K/H haem domains, and their endogenous amino acid sixth ligands remained in place. However, NO complexes were formed for A264M/C/Q proteins, with Soret shifts to 435, 433 and 433.5 nm respectively (see Figure 2B for A264Q–NO complex spectrum). Thus NO can displace any endogenous sixth ligand provided by the Met644, Cys644 or Glu644 side chains in the ferric forms of these enzymes.
Catalytic properties of Ala<sup>264</sup> mutant flavocytochromes P450 BM3

To examine effects of Ala<sup>264</sup> mutations on P450 BM3 catalytic properties, steady-state analysis of fatty-acid-dependent NADPH oxidation was done (see the Experimental section). Data for the reactions with dodecanoate and arachidonate are presented in Table 2. Rate ($k_{cat}$) and apparent substrate affinity ($K_m$) constants for WT BM3 are consistent with previous values (e.g. [13,14]), but $k_{cat}$ values for all mutants were substantially lower than for WT. The kinetic constants for the A264K/H/C mutants were second-order with respect to fatty acid concentration for both dodecanoate and arachidonate. Although this was expected for the A264K/H enzymes (where endogenous lysine/histidine ligands are not displaced on haem reduction), the data for the A264C variant were surprising in the light of optical studies suggesting that the Cys<sup>264</sup> side chain did not interact with haem iron, and that some substrate-dependent shift of haem iron spin-state equilibrium (towards HS) occurred on arachidonate binding. For the A264M mutant (where a more complete arachidonate-dependent spin-state shift was observed), the $K_m$ value indicated high affinity for the fatty acid (10.3 μM), but $k_{cat}$ was substantially lower than WT (115 min<sup>−1</sup>). A264M turnover with dodecanoate was also much slower than WT ($k_{cat} = 195$ min<sup>−1</sup>), although $K_m$ was also lower than WT (5.2 ± 0.8 μM). Apparent arachidonate affinity was also high for the A264Q flavocytochrome ($K_m = 1.86$ μM), and the $k_{cat}$ rather higher than for other mutants described in the present paper (754 min<sup>−1</sup>). However, A264Q-catalysed dodecanoate turnover was also second-order with respect to fatty acid concentration, albeit with a superior rate constant to that for the A264K/H/C mutants (Table 2). Collectively, these data demonstrate clearly that fatty acid oxidation is compromised in all Ala<sup>264</sup> mutants, and that the position of the relevant amino acid 264 side chain (even in enzymes or subpopulations thereof where the residue does not ligate the haem iron) probably interferes with catalytically productive substrate docking. Steady-state cytochrome c reduction (requiring electron transfer from FMN, and hence preceding reactions of FAD reduction by NADPH and inter-flavin electron transfer) was investigated for all mutant flavocytochromes, and at near-saturating concentrations of NADPH (500 μM) and cytochrome c (200 μM). In all cases, rates of > 100 s<sup>−1</sup> were determined, consistent with the value determined previously for the WT BM3 ($k_{cat} = 109$ ± 4 s<sup>−1</sup>), and demonstrating clearly that electron transport from NADPH through to the FMN cofactor (reduction of cytochrome c occurs near-exclusively from the FMN) is not significantly affected in Ala<sup>264</sup> mutant reductase domains and thus cannot explain decreased fatty acid oxidation kinetics [12].

To establish that fatty-acid-stimulated electron transfer in WT and Ala<sup>264</sup> mutant flavocytochromes was linked to substrate hydroxylation, products from turnover of dodecanoate were characterized. For WT BM3, all substrate was converted into monohydroxylated products (ω−1 to ω−3 hydroxylation of dodecanoate), as described previously [38]. Under the same conditions, small amounts of the same hydroxydodecanoate products were obtained from A264Q (∼5 % product formed), A264E (∼8 %) and A264M (∼5 %) mutants, whereas almost 20 % product formation was observed for A264C. Thus most product formation was observed for the A264C flavocytochrome, in which there appears to be no haem iron co-ordination by Cys<sup>264</sup>. Despite a low rate of substrate-dependent NADPH oxidation, a substantially higher proportion of product is formed in this mutant, possibly due to less steric interference with fatty acid docking close to the haem by the relatively small Cys<sup>264</sup> side chain (compared with Gln/Glu/Met<sup>264</sup> residues). Levels of product formation in the A264M/C/Q/E mutants thus do not correlate with extent of spin-state perturbation induced by fatty acid binding (although formation of HS haem iron was correlated previously with a favourable shift in WT BM3 haem iron potential and electron transfer to the haem iron [38]), or with the apparent $k_{cat}$ values with dodecanoate. Thus it is concluded that dodecanoate hydroxylation is much more uncoupled from NADPH oxidation in the A264M/Q/E mutants than in A264C, despite A264C’s much lower rate of fatty-acid-stimulated NADPH oxidation. In turn, A264C is substantially more uncoupled than WT BM3. The A264K/H mutants are inactive fatty acid hydroxylases, consistent with other studies showing that the Lys/His<sup>264</sup> side chains are not displaced by substrate binding or haem reduction.

### Potentiometric studies on Ala<sup>264</sup> mutants

To establish haem iron midpoint reduction potentials for the Ala<sup>264</sup> mutants, and to assess whether substrate-dependent modulation of haem iron potential occurs (as in WT BM3), spectroelectrochemical titrations of both ligand-free and arachidonate-bound haem domains were carried out, as described previously [13,30]. Consistent with previous data, a substantial increase (109 mV) in haem potential was determined for arachidonate-bound WT BM3 domain [30,39]. This elevation of potential (probably together with structural reorganization) probably facilitates FMN-to-haem electron transfer in the substrate-bound flavocytochrome [11]. For the A264C/Q haem domains, arachidonate binding also induces large increases in HS ferric haem iron content. The haem redox potentials for substrate-free A264Q and A264C haem domains were identical, within error, with that for WT, and arachidonate binding induced large increases in haem potential in both cases (361 and 145 mV respectively) (Table 3). The reduced states of substrate-free and arachidonate-bound forms of the A264Q/C mutants have Soret maxima at 410/411 nm respectively (Figure 3). These absorption values are similar to that for WT BM3 haem domain and indicate retention of thiolate co-ordination in the ferrous state. For A264C, the redox potential change is of similar magnitude to that for WT BM3 haem domain [30,39], but the increase in potential for A264Q is substantially greater than determined previously for WT or other P450 BM3 mutants.

The redox potential for A264M in the substrate-free form was also substantially more positive than for WT haem domain (by 172 mV), and was not perturbed significantly by arachidonate binding, even though there was substantially greater HS haem iron content in the arachidonate-bound form of the ferric A264M domain than in the substrate-free form (Table 3 and Figure 3).

### Table 3 Haem iron redox potentials for WT and Ala<sup>264</sup> mutant BM3 haem domains

<table>
<thead>
<tr>
<th>P450 BM3 haem domain</th>
<th>Substrate-free</th>
<th>Arachidonate-bound</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>−392 ± 5</td>
<td>−283 ± 4</td>
</tr>
<tr>
<td>A264H</td>
<td>−412 ± 8</td>
<td>ND</td>
</tr>
<tr>
<td>A264K</td>
<td>−420 ± 8</td>
<td>ND</td>
</tr>
<tr>
<td>A264Q</td>
<td>−396 ± 5</td>
<td>−35 ± 4</td>
</tr>
<tr>
<td>A264M</td>
<td>−220 ± 8</td>
<td>−227 ± 7</td>
</tr>
<tr>
<td>A264C</td>
<td>−385 ± 5</td>
<td>−240 ± 6</td>
</tr>
</tbody>
</table>

Haem iron potentials were determined as described previously and for substrate-free and arachidonate-bound proteins [22,30]. Data are midpoint reduction potentials relative to the SHE (standard hydrogen electrode). Data for the A264E mutant are from [22]. ND indicates not determined for the A264K/H mutants, for which no modulation of haem spectrum was observed on binding of fatty acids.
substrate-free A264M, the reduced species has a Soret maximum at 410 nm and a distinct shoulder at 435 nm, with two visible region bands at 539 and 562 nm. The ferrous arachidonate-bound A264M mutant has its maxima at the same wavelengths as the substrate-free reduced form, but with decreased Soret intensity in the 435 nm shoulder. The 410 nm Soret and a distinct shoulder at 435 nm, with two visible region bands at 529, 558 and 568 nm. The species with Soret at 424 nm correlates with the A264H data (and with $pK_a$ titration, see below), indicating Lys-Fe(II)-Cys thiol co-ordination for this proportion of the enzyme. The 445 nm species is attributed to a Lys-Fe(II)-Cys thiolate co-ordinated form.

To clarify the nature of the mixed species observed in the ferrous A264K mutant, a $pK_a$ titration was performed. Enzyme, in an anaerobic environment, was reduced with sodium dithionite at pH 5.5 before titration with NaOH. The starting spectrum collected at pH 5.5 had its Soret at 423 nm, with a small shoulder at 445 nm, and with visible region bands at 565 and 536 nm. Elevation of pH resulted in a marked increase in intensity of the long-wavelength Soret shoulder, accompanied by decreased intensity of the 423 nm band. Visible region features shifted to 557 and 524 nm. We consider that the spectral shift observed for ferrous A264K reflects conversion from a species with near-complete thiol proximal co-ordination (at pH 5.5) into one with mixed thiol/thiolate co-ordination at pH 9. The conversion was reversible, and pH-dependence of spectral changes was fitted using the Henderson–Hasselbalch equation to give a $pK_a$ value of 6.50 ± 0.04 (Figure 4). Thus reversible protonation/deprotonation of the fifth ligand was observed, as seen in our previous work on CYP121 [36].

Spectroscopic analysis of BM3 A264 variants

Spectroscopic studies of the A264 mutant haem domains were performed to define more clearly the nature of haem iron co-ordination in the solution states of the proteins, and to enable firm conclusions to be drawn relating to any differences between haem iron co-ordination states in solution and crystal forms.
EPR analysis

EPR spectroscopy reports on the spin state of ferric haem iron, and on the nature and orientation of haem iron ligands. For the various BM3 A264 mutants, EPR could provide important data confirming, or otherwise, the presence of novel amino acid side-chain ligands to the haem iron and proportions of such species populated. Previous studies confirmed fatty acid substrate-dependent co-ordination of A264E BM3 haem iron by Glu264, and substrate-independent co-ordination by His264 and Lys264 in A264H/K mutants [22–24]. EPR spectra for WT and A264M/C/Q haem domains are overlaid in Figure 5. The $g$-values are indicated for features in each of the spectra. Microwave power was 2 mW, modulation amplitude was 10 G and temperature was 10.8 K, and proteins were at concentrations of 400 μM (WT), 450 μM (A264C), 520 μM (A264M) or 745 μM (A264Q).

For A264Q, the EPR spectrum is consistent with a LS ferric haem species. The $g$-values were distinct from those for WT BM3 haem domain, with features at 2.48 ($g_x$), 2.25 ($g_y$) and 1.90 ($g_z$), compared with 2.42, 2.26 and 1.92 for WT BM3. The $g$-values for A264Q are similar to those for A264K, which also contains a single set of rhombic features at 2.47, 2.26 and 1.91, thus suggesting that Gln264 occupies the sixth ligand position on the haem iron in A264Q under these conditions (i.e. at 10 K) [22]. Although these data cannot categorically define the nature of the ligating atom as an amide oxygen or nitrogen from the Gln264 side chain, it is likely to be the oxygen (see below).

For the A264M domain, there is a complex mixture of signals, indicating both HS and LS haem species (see the legend to Figure 5). A minor HS species occurs with $g$-values at 7.98, 3.62 and 1.71, and is assigned to a penta-co-ordinated haem iron with a cysteinate fifth ligand and the sixth aqua ligand displaced. Of the four LS species visible, the one with $g$-values at 2.43, 2.25 and 1.92 is similar to that for the substrate-free WT domain, and is thus assigned to a hexa-co-ordinated LS species with water as sixth ligand. The species with $g$-values at 2.53, 2.25 and 1.89, and at 2.47, 2.25 and 1.92, are assigned to novel LS haems with Cys-Fe-Met ligation. These assignments are consistent with previous EPR assignments for P450cam in complex with sulfur donor ligands [40]. Specifically, $g$-values of 2.5, 2.27 and 1.89 were observed for P450cam bound to dimethyl sulfide, compared with 2.42, 2.25 and 1.92 for the dimethyl disulfide complex, and 2.44, 2.25 and 1.91 for the aqua-co-ordinated LS resting form [40]. A further set of A264M $g$-values is observed at 2.39, 2.25 and 1.98. Similar heterogeneity (i.e. three distinct LS cysteinate-co-ordinated species) was observed previously in studies of P450cam bound to propane-1-thiol [40]. Addition of substrate (500 μM arachidonate) to the A264M mutant provided data further consistent with these assignments, with a decreased complexity of the EPR signal, and an increase in the HS component (with $g_z$ at 7.98) at the expense of the aqua-co-ordinated component (with $g_z$ at 2.43). EPR signals are sensitive to the orientation as well as to the nature of haem iron ligands. Thus the complex mixture of A264M LS species may be a consequence of different orientations of the distal thioether and/or proximal cysteinate ligands. Since orientation of the cysteinate is conserved in crystal structures of WT and all Ala264 mutants, we consider heterogeneity of the distal ligand position to be more likely in A264M.

For the A264C domain, there is also a more complex population of species than in WT haem domain (Figure 5). Apart from the component assigned to LS, aqua-co-ordinated (2.42, 2.25 and 1.92) and a small proportion of HS penta-co-ordinated haem iron ($g$-values as for the comparable A264M species), there are other sets of $g$-values (specifically 2.47, 2.25 and 1.91, and 2.37, 2.25 and 1.94) that suggest interactions between the Cys264 side chain and the haem iron. The species with $g_z$ at 2.37 is comparable with that for P450cam in complex with the dithiothreitol anion, and thus suggests bis-thiolate co-ordination of BM3 haem iron [40]. The species with $g_z$ at 2.42 is comparable with EPR features for the 2-mercaptoethanol- and methanethiol-bound forms of the cysteinate-co-ordinated Caldariomyces fumago chloroperoxidase enzyme, and for propanethiol-bound P450cam, and may thus relate to species with thiol distal co-ordination [40,41]. However, EPR data indicative of Cys264 interaction with haem iron in A264C are at odds with crystal structural data, and possible explanations are considered in more detail below.

MCD studies

MCD is an important tool for characterizing iron co-ordination in haemoproteins [42]. MCD spectra were recorded in both near-UV–visible (300–700 nm) and NIR (700–1400 nm) regions to obtain further data on haem co-ordination states of Ala264 mutants. In particular, the position of a LS porphyrin-to-ferric haem iron CT (charge-transfer) band in the NIR MCD region is an important reporter of alterations to haem ligation state [43].

Near-UV–visible MCD spectra for WT and A264M/C/Q mutants are shown in Supplementary Figure S1 at http://www.

Figure 5 Perpendicular mode EPR spectra of WT and Ala264 mutant P450 BM3 haem domains at X-band frequency

The spectra for WT and A264C/M/Q BM3 haem domains are overlaid, and $g$-values are indicated for features in each of the spectra. Microwave power was 2 mW, modulation amplitude was 10 G and temperature was 10.8 K, and proteins were at concentrations of 400 μM (WT), 450 μM (A264C), 520 μM (A264M) or 745 μM (A264Q).
BiochemJ.org/bj/417/bj4170065add.htm. These reveal differences in band positions and intensities between WT and Ala264 mutants. Thiolate (i.e. cysteinate) co-ordination is indicated by the relative band positions and their low intensities compared with those for other LS haemoproteins with different co-ordinating ligand(s). For the A264Q/C mutants, the haem is predominantly LS (as for WT BM3) with CT bands at 575.9/577.1 nm, compared with 575.7 nm for WT BM3. In contrast (and as observed in the electronic absorption spectra), the A264M mutant has the CT band shifted to 573.5 nm, consistent with a considerable proportion of HS haem iron. Differences consistent with a greater proportion of HS haem in A264M are also evident from the derivative feature in the 600–700 nm range. WT and A264C/Q mutants have minima between 656.5 and 657.5 nm, but the A264M minimum is shifted to 645.5 nm, and is of much greater intensity than for WT and the other mutants. In the NIR MCD region, the CT,LS peak is an important marker for the haem iron co-ordination state. In the WT haem domain, the peak is at 1077 nm, consistent with previous studies [22,44]. The CT,LS peak is also at 1077 nm for A264C, but is shifted to 1084 and 1086 nm for the A264M and A264Q mutants respectively (Supplementary Figure S1). As can be seen for the substrate-bound HS form of WT BM3 haem domain, the CT,LS band is considerably diminished in intensity, with the development of a CT band at ~835 nm. A small HS feature is also observed for A264C, and the A264M variant has a larger amount of the HS feature, again consistent with optical studies. Conclusions from these room temperature (20 ± 2 °C) MCD studies are that there is possibly little haem iron co-ordination by Cys264 in the A264C variant, but that both Met264 and Glu264 ligate, at least in a proportion of the enzyme, in the respective A264M and A264Q mutants. For A264M (again consistent with other spectroscopic studies reported here), there is evidence for both penta-co-ordinate HS and hexa-co-ordinate LS species, all with cysteinate as proximal ligand. There are clearly two hexa-co-ordinate forms, which we assign to either Met264 or water ligated in the distal position. For the A264Q mutant, MCD studies demonstrate an almost exclusively LS haem, with retention of the cysteinate proximal ligand and either water or Glu264 in the distal position. The position of the NIR CT,LS band is at ~1086 nm for A264Q. In previous studies of the A264E mutant, this feature shifted from 1080 nm (partial occupancy of Glu264 as the sixth haem ligand) to 1085 nm in the arachidonate-bound form, where arachidonate binding induced a near-complete switch to a Cys-Fe(III)-Gln haem iron ligand set [22,23]. The similar position of this feature in A264Q is consistent with a considerable proportion of this enzyme adopting a Cys-Fe(III)-Gln ligand set, and would also suggest that the amide oxygen atom of Glu264 is the sixth ligand to the haem iron.

RR characterization

RR spectroscopy provides important information on vibrational frequencies of bands within a P450 haem, and reports on haem iron oxidation, spin and co-ordination states [45,46]. Data were collected for Ala264 mutant haem domains with laser excitation at 406 nm, close to the Soret maximum. In all cases, the ν4 vibration state marker band was centred at 1371/1372 cm⁻¹, consistent with previous studies on WT BM3 haem domain and confirmatory of ferric haem iron in each Ala264 mutant [14]. In all cases, substrate (500 μM arachidonate) binding did not alter the ν4 position, confirming retention of ferric haem iron. The ν3 feature is a convenient haem iron spin-state marker band, and for WT BM3 haem domain is resolved by curve fitting into two features at 1481 cm⁻¹ (HS) and 1500 cm⁻¹ (LS), with the LS species predominant [47,48]. On substrate addition, the balance changes in favour of the HS ν3 species, consistent with optical data. The A264K/H mutants have ν3 at 1495 cm⁻¹ in both substrate-free and arachidonate-bound forms, confirming retention of the LS state and the nitrogenous sixth ligand in the substrate-bound ferric forms. A264Q haem domain in its substrate-free state has a major ν3 feature at 1500 cm⁻¹, but a small shoulder develops at 1480 cm⁻¹ on arachidonate binding, indicative of a minor proportion of HS haem iron and consistent with optical titration data. For A264M, ν3 features are at 1482 cm⁻¹ (minor) and 1500 cm⁻¹ (major) for the as-purified protein, with these features becoming roughly equal in intensity for arachidonate-bound protein. Structural data for A264M haem domain indicate that some fatty acid remains bound through the purification process (see below), consistent with the RR data here and with A264M Kd values for fatty acid binding being lower than those for WT (Table 2). For A264C, ν3 values are ~1501 cm⁻¹ for substrate-free haem domain, with (as for WT) a shift in equilibrium towards a 1482 cm⁻¹ species on arachidonate binding. Collectively, RR data confirm that WT and all Ala264 mutants are thiolate-co-ordinated ferric proteins in their resting state, and that all but A264M are predominantly LS in absence of exogenous substrate. Arachidonate binding also leads to accumulation of penta-co-ordinate HS signals for the A264C/Q mutants. Other RR features reporting on perturbations to haem ring planarity and conformations of haem substrate groups are discussed further in the Supplementary Online Data. RR spectra for WT and the A264M/C/Q/K/H mutants are shown in Supplementary Figure S2 at http://www.BiochemJ.org/bj/417/bj4170065add.htm, and frequencies and intensities of the key vibrational bands are annotated in Supplementary Table S3 at http://www.BiochemJ.org/bj/417/bj4170065add.htm.

Structural analysis of A264M, A264Q and A264C haem domains

Studies of the BM3 A264E/K haem domains revealed that both substrate-free and palmitoleate-bound forms of these mutants crystallized in a conformation previously considered as that populated by WT BM3 in its substrate-bound form [23,24]. The lower Kd values determined for fatty acid binding to A264E (compared with WT BM3) suggested that the substrate-bound conformation adopted by this mutant was that with higher affinity for fatty acids (as opposed to substrate binding to the substrate-free form and then inducing a conformational ‘switch’) [22]. Structural studies of the A264H mutant showed this haem domain occupied the substrate-free conformation (see Supplementary Figure S3 at http://www.BiochemJ.org/bj/417/bj4170065add.htm). This conformation was the only one compatible with distal co-ordination of haem iron by the His264 side chain [24]. Here, we crystallized the A264M/C/Q haem domains, and collected X-ray diffraction data to a resolution of 2.5, 2.1 and 2.0 Å (1 Å = 0.1 nm) respectively. All crystallized in the same space group (P212121) as the previously determined A264E/K haem domains, and were in the substrate-bound conformation [23,24]. Data collection and final refinement statistics for the new mutant structures are presented in Supplementary Table S1.

Analysis of haem environment in the A264M haem domain demonstrated clearly that the Met264 side chain co-ordinated the haem iron, creating a novel hexa-co-ordinated Cys-Fe-Met protein (Figure 6). Our data are consistent with spectroscopic studies and confirm that this unprecedented haem iron ligand set exists in both solution and crystal states of A264M haem domain. EPR (at 10 K) and room temperature optical studies indicate that both ‘Met264-on’ forms (i.e. those where Met264 ligates the haem iron), a pentaco-ordinate HS species and a hexa-co-ordinate aqua-co-ordinated LS form exist in equilibrium. The predominance of the
In agreement with low fatty acid $K_d$ values for A264M, there was clear electron density corresponding to a bound fatty acid substrate in one of the two molecules in the asymmetric unit. This was modelled as palmitate, consistent with previous work demonstrating co-purification of palmitate with two other fatty acid hydroxylases purified from *E. coli*, namely *Bacillus subtilis* P450 Biol (CYP107H1) and the peroxide-driven P450 BM3 from the same bacterium [28,49]. The ω-terminal region of the bound fatty acid interacts with the Phe$^{37}$ side chain, as seen for palmitoleate-bound WT BM3 haem domain [50]. However, for both molecules in the asymmetric unit, haem iron ligation by the methionine and overall protein conformation are identical and thus appear independent of the presence of the fatty acid. From optical spectra, the purified A264M flavocytochrome had a HS content similar to that of the A264M haem domain, confirming that high affinity for fatty acids is retained in intact A264M enzyme.

The crystal structure of the A264C haem domain shows the Cys$^{264}$ side chain above the haem plane, within van der Waals distance of both Phe$^{37}$ and the sixth water ligand (Figure 6). It makes no stable hydrogen-bonding interactions with any other residue or water molecules, and there was no evidence of oxidation of the cysteine thiol from the electron density. The picture of a single ligation state, as observed for both A264C haem domains in the asymmetric unit, is somewhat at odds with the complexity and heterogeneity of the EPR spectrum. It is possible that the alternative species are only minor populations within the crystal, making their detection difficult. These alternative forms could correspond to various oxidized forms of the Cys$^{264}$ sulfur (e.g. sulfenic, sulfinic or sulfonic forms) created following sulfur reactions with oxy-haem intermediates. Each of these species could contact the haem iron or its sixth aqua ligand and perturb the EPR signal. Oxygen atoms from putative oxidized cysteine species may also be disordered in crystals, complicating further their detection. Although direct Cys$^{264}$ ligation of haem iron might occur in solution, this would require major structural reorganization of the protein. However, the plasticity of the BM3 haem domain is well known from preceding structural studies [22–24]. Similar to most Ala$^{264}$ mutants, both A264C haem domains within the asymmetric unit are in the substrate-bound conformation, although no fatty acid substrate was detected in either molecule in this case.

In contrast with the A264M/C mutants, which show no significant heterogeneity in ligation state in the crystal structures, dual occupancy for the Gln$^{264}$ side chain in the A264Q mutant was observed (Figure 6). These conformations correspond to ‘Gln-on’ and ‘Gln-off’ states, with respect to co-ordination of the haem iron by the Gln$^{264}$ side chain. This is analogous to the situation for the A264E mutant, as the Gln-off state has the Gln$^{264}$ side chain interacting with the Phe$^{37}$ side chain [22,23]. Similarly to the A264E mutant, we have modelled the Gln-on state as ligating through the amide oxygen. This assignment remains tentative on the basis of X-ray crystallographic data alone (which cannot distinguish between oxygen or nitrogen ligation at this resolution). EPR data are similarly inconclusive, since there is no evidence of any line splitting that could be indicative of a nitrogen ligand. As for previously determined haemoprotein structures with amide ligands, e.g. the penta-co-ordinated H175Q mutant of cytochrome c peroxidase (CCP) and the hexa-co-ordinated *Rhodobacter sphaeroides* haemoprotein SHP (*sphaeroides* haem protein), we have modelled A264Q ligation to occur via the oxygen atom, similar to known ligation patterns of amide compounds with haems [51,52]. Co-ordination of haem iron by nitrogen in the BM3 A264Q mutant would require deprotonation of the amide nitrogen [50]. SHP is the only known naturally occurring example of a haem amide ligation. In this case,
the haem iron axial ligands are His47 and Asn68. In ferric SHP, the Asn68 amide co-ordinates the haem iron, but, on reduction, this sixth ligand dissociates to leave a penta-co-ordinated ferrous haem iron bound only by a His47 axial ligand [52]. Our data for the BM3 A264Q mutant are thus the first reporting a hexa-co-ordinate amide-ligated haem with cysteinate as the fifth ligand, and the first example of a hexa-co-ordinated haem protein with glutamin as an axial ligand. In the Gln-off state, a water molecule replaces the Gln264 amide as haem sixth ligand. The A264Q haem domain is also in the substrate-bound conformation, but again without substrate bound.

The A264M/C/Q haem domains crystallize in the substrate-bound conformation, as seen for A264E and for palmitoleate-and other substrate-bound forms of WT BM3 haem domain
[23,50]. The substrate-bound form is likely to be one of (at least) two major solution-state conformers of the BM3 haem domain, and possibly the conformer with higher affinity for fatty acids [22]. Co-ordination of haem iron by Met264 and Glu264 side chains clearly occurs in the substrate-bound conformer in the A264M/Q crystals. Apparent differences in proportions of the sixth ligand co-ordinated in these mutants between solution state, crystal and (low temperature) EPR forms may result from changes in the equilibrium of conformational states under these different conditions. Heterogeneity observed in the EPR spectra of A264M/C/Q mutants also suggests different ligation states and conformations of co-ordinating ligands. Covalent (oxidative) modification of the Cys264 side chain, which is too short to reach the haem iron in the substrate-bound conformation crystallized, might also occur (Figure 6). For A264M, the hydrophobic Met264 side chain could also interact with the $\omega$-end of the fatty acid substrate (at least in the room temperature solution state) to enable development of HS haem iron.

Concluding remarks

Our work reveals novel haem iron ligand sets, created by engineering haem iron co-ordinating residues at position 264 in the I helix region of P450 BM3. The first clear structural and/or spectroscopic evidence for Cys-Fe-Met and Cys-Fe-Gln haem iron ligation states is provided, and characterization of reduced forms of these species (and of other novel hexa-co-ordinated species) is presented. The A264M/C/Q/E mutants retain fatty acid hydroxylase activity, although activity is diminished and NADPH oxidation is substantially uncoupled from dodecanoate hydroxylation in all cases. The A264K/H mutants were inactive, consistent with essentially irreversible co-ordination of haem iron by Lys/His264 side chains. Substrate-dependent conversion of P450 haem iron does not correlate well with rate of fatty acid hydroxylation in A264M/C/Q/E mutants, and lack of active-site steric hindrance from the smaller Cys264 side chain may underlie the more efficient coupling of NADPH oxidation to substrate hydroxylation in the A264C enzyme. The A264M mutant has higher affinity than WT for fatty acids (lower $K_C$ values), consistent with its co-purification with lipid (probably palmitate) from E. coli, as is evident from its crystal structure. The combination of structural and spectroscopic analyses highlight differences in occupancy (and likely orientation for A264M) of the novel haem iron sixth ligands depending on temperature and solution, frozen or crystalline state of the proteins, which is likely to be testament to the different conformational states that can clearly be occupied by this P450.

In conclusion, our data present detailed structural/spectroscopic characterization of novel haem iron ligation sets in at least the A264M/Q mutants of BM3. In detailing the spectroscopic features of the BM3 A264 mutants, we provide data that will enable identification of such systems as and when they are observed in Nature.

ACKNOWLEDGEMENTS

A.W.M. thanks the Royal Society for the award of a Leverhulme Trust Senior Research Fellowship. D.L. is a Royal Society University Research Fellow.

FUNDING

This work was supported by the Biotechnology and Biological Sciences Research Council [grant numbers BB/C006879/1, BB/F00883X/1].

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SUPPLEMENTARY ONLINE DATA

Novel haem co-ordination variants of flavocytochrome P450 BM3

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Table S1 Data collection and final refinement statistics for P450 BM3 A264 mutants

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<th>PDB code</th>
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<th>3EKD</th>
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Table S2 LS g-values from EPR spectra of the ferric ligand-free forms of P450 BM3 WT and A264 mutant haem domains

<table>
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<th>BM3 haem domain</th>
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<th>g₂</th>
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<td>A264H †</td>
<td>2.47</td>
<td>2.26</td>
<td>1.91</td>
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Table S3 RR assignments for ferric forms of P450 BM3 WT and Ala264 mutant haem domains

Peak assignments (to the nearest 1 cm⁻¹) in the resonance Raman (RR) spectra were according to previous publications [2–5]. Values stated are for the substrate-free proteins. The ν₁ band is the major feature in the spectrum. This is an oxidation state marker and indicates retention of ferric haem iron in all samples. The ν₁ν₁ band in the high-frequency region of the spectrum is associated with C–C stretching and is affected by the electronic conjugation of the porphyrin and the vinyl groups. It thus reports on the in-plane asymmetry of the haem ring [4,5]. A more domed or ruffled haem domain should give rise to a stronger ν₁ν₁ signal. For the WT haem domain, the ν₁ν₁ band is at 1565 cm⁻¹, with a small decrease in intensity on substrate binding. Its diminished intensity in the A264K/H mutants (its presence was not detected in the curve-fitting procedure) suggests that the complete co-ordination of the haem iron by the nitrogenuous Lys/His264 ligands has resulted in a more planar haem domain.

<table>
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<tr>
<th>RR peak assignment</th>
<th>WT</th>
<th>A264K</th>
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**EXPERIMENTAL**

**Ligand-binding titrations**

Titrations were performed by addition of small volumes of substrate/ligand (typically 0.1–0.4 μl, to a maximum total volume of ~5 μl, i.e. 0.5% of the total reaction volume) across the concentration range required for complete conversion into the ligand-bound form. Spectra were recorded at each titration point, and difference spectra were computed by subtraction of the spectrum for the ligand-free form from the relevant ligand-bound spectral species. The maximal overall absorption shift induced at each point was determined by subtraction of the absorption value at the trough from that at the peak in each difference spectrum (using the same wavelengths in each case). Absorption values calculated in this way were plotted against the relevant ligand/substrate concentration, and data were fitted to the relevant equation. For weaker binding substrates/ligands, data were fitted to a hyperbola. For substrates/ligands with \(K_d\) values not substantially greater than the concentration of enzyme used in the assay, data were fitted instead to a quadratic function (eqn 1), as described previously [1]:

\[
A_{obs} = \left(\frac{A_{max}}{2Et}(S + Et + K_d)\right) - \left[(S + Et + K_d)^2 - (4S \cdot Et)^{0.5}\right] \tag{1}
\]

where \(A_{obs}\) is the absorption shift determined at substrate/ligand concentration \(S\), \(A_{max}\) is the maximal absorption shift obtained at saturation with the substrate/ligand, \(Et\) is the total enzyme concentration used, and \(K_d\) is the dissociation constant for the substrate/ligand–enzyme complex.

**Potentiometric analysis**

Redox potentials for the ferric/ferrous transition of the P450 BM3 haem iron were determined for the A264M, A264C and A264Q mutant haem domains in their substrate-free and arachidonate-bound forms. Enzymes were titrated with reductant (sodium dithionite) and oxidant (potassium ferricyanide) in the presence of mediators (to expedite communication between oxidant/reductant, enzyme and electrode) and to various states of reduction of the haem iron. The applied potential (measured via a calomel electrode immersed in the enzyme solution) and UV-visible absorption spectrum were recorded simultaneously. A fibre optic probe (Varian Instruments) was immersed in the enzyme solution and linked to a Cary UV-50 Bio spectrophotometer outside the glove box in order to record absorption spectra (typically 750–250 nm). Absorption values (in the Soret region and reflecting the maximal overall absorption change between the ferric and ferrous forms of the P450s) were plotted against the relevant applied potential, and data were fitted using the Nernst equation (using Origin software, OriginLab), to determine the midpoint reduction potential for the P450 haem iron in each case. Mediators used were PMS (phenazine methosulfate) (2 μM), HNQ (2-hydroxy-1,4-naphthoquinone) (7 μM), MV (Methyl Viologen) (0.3 μM) and BV (Benzyl Viologen) (1 μM), to mediate in the range from +100 to −480 mV. Redox titrations were performed at 25 °C and usually completed in a period of 3–4 h. The electrochemical potential of the solution was measured using a Hanna pH 211 meter coupled to a Pt/calomel electrode (ThermoRussell). The electrode was calibrated using the ferric/ferrous EDTA couple as a standard (108 mV). A factor of 244 mV was used to correct relative to the standard hydrogen electrode. Redox potentials were measured for mutant haem domains in their substrate-free state and in the presence of the substrate arachidonate at near-saturating levels (66 μM).
Novel P450 BM3 haem co-ordination mutants

Figure S2  RR spectra for the ligand-free ferric forms of P450 BM3 WT and Ala264 mutant haem domains

RR spectra were collected as described in the Experimental section of the main text. Key features in the spectra are indicated. Assignments and peak positions for these (and other) features are presented in Table S3.

Figure S3  Conformational properties of the P450 BM3 A264H and A264M haem domains

(A) A superimposition of the distinct P450 BM3 conformations illustrated by the A264H mutant structure (for substrate-free conformation) and the A264M structure (for substrate-bound conformation). The view on the left has the A264H structure coloured cyan, whereas the A264M structure is in grey. The view on the right is similar, but has A264M in green and A264H in grey. The A264M-bound palmitate is shown in green spheres for the right-hand panel, whereas both panels have haem presented as red sticks. The major changes between both conformations occur in the positions of the F and G α-helices, as indicated by arrows. (B) Detail of the overlay between A264H (carbons in yellow) and A264M (carbons in blue) haem domain structures, with overlay based on residues 395–405. Palmitate is shown in magenta and haem cofactor is coloured red. A segment of the I-helix (residues 263–268) and selected residues contacting the substrate in A264M (monomer A) are shown. The RMSD (root mean square deviation) for all Cα carbons is 1.68 Å.

REFERENCES


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Received 4 June 2008/14 August 2008; accepted 22 August 2008
Published as BJ Immediate Publication 22 August 2008, doi:10.1042/BJ20081133

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